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Abstract: BACKGROUND: Epidermodysplasia verruciformis (EV) is a rare genodermatosis that is characterized by susceptibility to infection with specific human papillomavirus (HPV) genotypes. Among polyomaviruses, the novel Merkel cell polyomavirus (MCPyV) has been found in different epithelial skin neoplasias. OBJECTIVE: To examine whether EV is associated with cutaneous MCPyV infection. METHODS: We used MCPyV-specific PCR to study skin neoplasms of 6 congenital EV patients and of 1 patient with acquired EV. RESULTS: In all congenital EV patients, MCPyV DNA was found in carcinomas in situ, in invasive squamous cell carcinomas and in common warts. In 4 of these patients, the MCPyV-positive skin lesions were from different anatomic locations. In addition, 1 immunosuppressed patient suffering from acquired EV harbored MCPyV DNA in 2 common warts. In contrast, 7 normal skin samples tested negative for MCPyV DNA. Only 2 out of 24 carcinomas in situ (8.3%) and 2 out of 30 common warts (6.7%) from immunocompetent individuals were positive for MCPyV DNA. CONCLUSIONS: The strong association of EV-associated skin neoplasms with MCPyV suggests a unique susceptibility of EV patients to infections with MCPyV. Both MCPyV and EV-HPV may act as synergistic oncogenic cofactors in the development of EV-associated skin neoplasms.

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Detection of Merkel Cell Polyomavirus in Epidermodysplasia-Verruciformis-Associated Skin Neoplasms

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Key Words
Merkel cell polyomavirus · Human papillomavirus · Epidermodysplasia verruciformis · Cutaneous oncogenesis

Abstract
Background: Epidermodysplasia verruciformis (EV) is a rare genodermatosis that is characterized by susceptibility to infection with specific human papillomavirus (HPV) genotypes. Among polyomaviruses, the novel Merkel cell polyomavirus (MCPyV) has been found in different epithelial skin neoplasias. ***Objective:*** To examine whether EV is associated with cutaneous MCPyV infection. ***Methods:*** We used MCPyV-specific PCR to study skin neoplasms of 6 congenital EV patients and of 1 patient with acquired EV. ***Results:*** In all congenital EV patients, MCPyV DNA was found in carcinomas in situ, in invasive squamous cell carcinomas and in common warts. In 4 of these patients, the MCPyV-positive skin lesions were from different anatomic locations. In addition, 1 immunosuppressed patient suffering from acquired EV harbored MCPyV DNA in 2 common warts. In contrast, 7 normal skin samples tested negative for MCPyV DNA. Only 2 out of 24 carcinomas in situ (8.3%) and 2 out of 30 common warts (6.7%) from immunocompetent individuals were positive for MCPyV DNA. ***Conclusions:*** The strong association of EV-associated skin neoplasms with MCPyV suggests a unique susceptibility of EV patients to infections with MCPyV. Both MCPyV and EV-HPV may act as synergistic oncogenic cofactors in the development of EV-associated skin neoplasms.

Introduction

Epidermodysplasia verruciformis Lewandowsky-Lutz (EV) is a rare autosomal recessive genodermatosis associated with diminished cell-mediated immunity and high susceptibility to infection with specific human papillomavirus (HPV) genotypes [1, 2]. These HPV types are referred to as EV HPV types (also known as β-papillomaviruses) [3]. The most frequent EV HPV types are HPV type 5 and type 8 [4]. Uncontrolled HPV infections in EV patients result in flat warts and seborrheic-keratosis-like lesions arising during childhood or early adulthood. There is a high risk of developing carcinomas in situ (Bowen's disease) and invasive squamous cell carcinomas (SCC) later in life, mainly on sun-exposed skin [5–7].

The involvement of HPV in human skin cancer has been demonstrated first in patients suffering from EV [3, 8]. Of the various EV HPV types, particularly HPV-5 and

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Table 1. Patient characteristics

ID No.	Sex	Age years	Diagnosis	Localization	VP1	LT1	LT3	HPV	EV
1	F	80	SCC	temple	+	+	+	5	+
		80	AK	eyelid	+	+	+	–	
		81	BD, BCC	nose	–	–	–	5	
		81	BD	neck	–	+	+	5	
		82	BD	hand	+	+	+	5	
		82	BD	hand	+	+	+	5	
2	F	73	SCC	face	+	+	+	–	+
		73	SCC	face	+	+	+	5	
		73	SCC	face	–	–	–	5	
3	M	50	BD	back	+	+	+	5	+
		50	BD	back	+	+	+	5	
		50	BD	leg	+	+	+	5	
		50	BD	leg	+	+	+	5	
		50	BD	leg	–	+	+	5	
4	M	48	BD	face	+	+	+	5, 8	+
		48	AK	arm	+	+	+	5, 8	
		48	AK	arm	–	–	–	5, 8	
5	M	25	verruca	forehead	+	+	+	5	+
6	M	64	verruca	arm	–	–	+	n.d.*	–
7	M	34	verruca	neck	+	+	+	8	AEV
		37	verruca	arm	+	+	+	8	AEV

The data are from 6 patients (No. 1–6) suffering from various skin neoplasms associated with congenital EV. One additional patient (No. 7) with flat warts suffered from acquired EV (AEV). The age of each patient refers to the age at the time of histological diagnosis of any skin lesion. Suitability of DNA for analysis was confirmed by β-globin PCR. Amplifiable DNA was recovered in all study cases (not shown). To detect MCPyV sequences, PCR with 2 primer sets in the T antigen locus (LT1, LT3) and 1 in the VP1 gene (VP1) and direct sequencing of PCR products was performed. In all EV patients, we detected MCPyV sequences in at least 1 skin lesion. The results of HPV PCR and the HPV types identified by sequencing of the PCR products are indicated (HPV).

EV+ = EV genetically confirmed by mutation analysis of the *EVER* genes; EV– = EV genetically not confirmed by mutation analysis of the *EVER* genes, but clinical picture suggestive of EV; M = male; F = female; AK = actinic keratosis; BCC = basal cell carcinoma; BD = carcinoma in situ (Bowen's disease); verruca = common wart (verruca vulgaris); n.d. = no data. * Several EV HPV sequences were detected, but exact HPV types could not be determined by sequence analysis.

HPV-8 have been implicated in malignant transformation of EV lesions [9, 10]. The pathogenetic steps of EV-related carcinogenesis are not well understood [7]. In 2002, families with EV were found to have missense mutations in the *EVER1/TMC6* or *EVER2/TMC8* genes on chromosome 17q25 [2]. These genes encode cytoplasmic proteins that colocalize with calnexin, an integral membrane protein in the endoplasmic reticulum, and are likely to function as modifiers of ion channels and to be involved in signal transduction. It was proposed that EVER proteins act as restriction factors for EV-specific HPV in keratinocytes, and that EV represents a primary deficiency in intrinsic immunity against certain papillomaviruses [11]. Apart from classical, congenital EV with mutations in the *EVER* genes, an acquired form of EV (AEV) has recently been described in immunosuppressed patients [12]. Despite its rarity, EV was addressed in depth in the recent literature because it represents a model for virally mediated cutaneous oncogenesis [13]. However, a unifying pathogenetic theory for EV is still lacking, and other pathogens could contribute to cutaneous oncogenesis in EV as well.

Polyomaviruses are small, nonenveloped, double-stranded DNA viruses. They often persist as latent infections, but are potentially oncogenic and may produce tumors upon reactivation, e.g. in immunocompromised hosts. The majority of Merkel cell carcinomas (MCC) were found to be infected with a novel human skin tropic polyomavirus, aptly named Merkel cell polyomavirus (MCPyV) [14]. In addition, MCPyV DNA was detected in nonmelanoma skin cancer (NMSC) lesions of both immunosuppressed and immunocompetent individuals [15–18].

In this study we analyzed if EV could be associated with an increased susceptibility not only to EV HPV, but also to human polyomaviruses, particularly to the potentially oncogenic MCPyV [19]. We demonstrate that all 6 of 6 congenital EV patients harbor MCPyV DNA in their skin lesions. Our data lead us to suggest a possible involvement of MCPyV in skin cancer development in EV patients and underline the need for investigation of additional infectious agents associated with this disease.

Patients and Methods

Patients

The patient characteristics are summarized in table 1. The diagnosis of EV was based on characteristic clinical features, histological findings and in congenital EV patients by identification of mutations in the *EVER* genes [Burger et al., in preparation]. Patients No. 1 and 2 were siblings that have been described before [6]. At the time point of this study, patient No. 1 was alive, and we were able to confirm her EV genetically by mutation analysis [Burger et al., in preparation]. In her late 30s, she first developed hyper- and hypopigmented macules on both legs. Over the years, she developed numerous NMSC lesions, mainly SCC and basal cell carcinomas on sun-exposed skin areas. Her sister, patient No. 2, had died approximately 20 years before this study was conducted. Sim-

ilar to her sister, patient No. 2 had developed multiple NMSC lesions, first SCC on her face, but later also at other anatomic locations. Three archival invasive SCC from her face were available for analysis. Patient No. 3 was a 50-year-old Caucasian male suffering from multiple disseminated flat warts since early childhood. Over the years, he had developed numerous (more than 20) carcinomas in situ (Bowen's disease). We analyzed 5 of his carcinoma in situ lesions from various anatomic locations in this study. Patient No. 4, a 48-year-old Caucasian male, developed the classical EV symptoms with multiple precancerous and cancerous skin lesions during early adulthood. From patient No. 4, we could examine 1 carcinoma in situ and 2 actinic keratoses for the presence of HPV and MCPyV. Patient No. 5 was a 25-year-old male EV patient of Turkish origin who clinically suffered from multiple warts out of which we analyzed 1. EV patient No. 6 was a 64-year-old Caucasian male with a clinical history highly suggestive of congenital EV, although a mutation analysis of the *EVER* genes was not performed.

The medical records for all patients were reviewed and the following relevant information was extracted: date of birth, sex, date of EV diagnosis, EV mutation analysis, treatment regimens, date of diagnosis of skin lesions, histological diagnoses of skin lesions and HPV analysis. All skin biopsies were collected for diagnostic purposes. The biopsies were formalin fixed, paraffin embedded and processed according to standard procedures. All HE-stained histology slides were reviewed independently by a board-certified pathologist (M.P.) and a board-certified dermatopathologist (W.K.). Control skin biopsies including 7 normal skin samples, 24 carcinomas in situ and 30 common warts of otherwise healthy, immunocompetent patients were randomly selected from our archive (Kempf and Pfaltz Histological Diagnostics) [16]. These patients had never been treated with immunosuppressive drugs, nor did they suffer from immunodeficiencies or EV.

This study was approved by the ethics review board of the Canton of Zurich, Switzerland. Written informed consent was obtained from all living patients. The study was conducted with strict adherence to the Declaration of Helsinki Principles.

Detection of Polyomavirus DNA by PCR

DNA extraction and purification was performed as described, and DNA quality was confirmed by β-globin PCR [20]. For MCPyV detection, we used 2 primer sets targeting the T antigen locus (LT1, LT3) and 1 targeting the VP1 gene (VP1) of MCPyV, as described [14, 20]. For detection of BK virus (BKV) and JC virus (JCV), a nested PCR with P3-P4 and P1-P2 primers was performed as published [21]. To simultaneously detect the presence of KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), a standard PCR with a single primer pair detecting part of the VP1 region of both viruses was performed as described [21].

Detection of HPV DNA by PCR

For HPV detection, we used 3 different protocols, as described elsewhere [16]. To preferentially detect EV HPV types, we used a PCR protocol with the outer primer set CP62-CP69 and the internal nested primer set CP65-CP68 [22].

Sequence Analyses

Amplified MCPyV and HPV PCR products were purified prior to sequencing, using the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. PCR products were submitted for automated sequencing, using a Roche FLX genome sequencer (Microsynth, Balgach, Switzerland). The resulting MCPyV DNA sequences were aligned against the following reference sequences of the NCBI (National Center for Biotechnology Information) Entrez Nucleotide database, using the NCBI BLAST program gb|EU375803.1 Merkel cell polyomavirus isolate MCC350, and gb|EU375804.1 Merkel cell polyomavirus isolate MCC339. The nucleotide blast database was searched to determine the degree of homology of HPV PCR products to known HPV subtypes.

Statistical Analysis

MCPyV DNA prevalence was compared between EV lesions and control groups (carcinomas in situ, common warts) using the two-sided Fisher exact test. Statistics employed in this study were carried out using GraphPad Prism 5 software. $p < 0.05$ was considered to be statistically significant.

Immunohistochemistry

A monoclonal antibody (CM2B4) that specifically recognizes MCPyV large T protein was used for immunohistochemical detection of MCPyV, as described [23].

Results

Detection of MCPyV in EV Patients

In this study, we analyzed a total of 6 patients diagnosed with classical congenital EV. MCPyV DNA was detected in all 6 EV patients, in at least 1 skin lesion per patient (table 1; fig. 1). In patients No. 1–4, MCPyV DNA was detected in multiple skin lesions at various anatomic locations. DNA of the other 4 known human polyomaviruses – BKV, JCV, KIPyV and WUPyV – was not detected in any of the samples (not shown). In all EV patients, EV HPV types were detected, consistent with the diagnosis of EV (table 1).

In order to verify the specificity of the amplified MCPyV PCR products and to detect genomic variants of MCPyV, all MCPyV PCR products were sequenced. The VP1 product differed by up to 3 bp from the predicted MCPyV amplicon of the MCC350 virus genome (gb|EU375803.1 Merkel cell polyomavirus isolate MCC350) found in MCC. The LT1 and LT3 sequences differed by up to 6 bp in all cases.

All EV skin lesions positive for MCPyV LT1 DNA by PCR were also tested for expression of MCPyV large T protein by immunohistochemistry. We found 2 of the EV lesions containing isolated keratinocytes that showed a faint reactivity for MCPyV large T protein (online suppl. fig. 1, www.karger.com/doi/10.1159/000321880).

We conclude that MCPyV DNA is widely present in skin lesions of EV patients. We found MCPyV DNA only in 2 out of 24 carcinomas in situ (8.3%) and in 2 out of 30 common warts (6.7%) of healthy, immunocompetent pa-

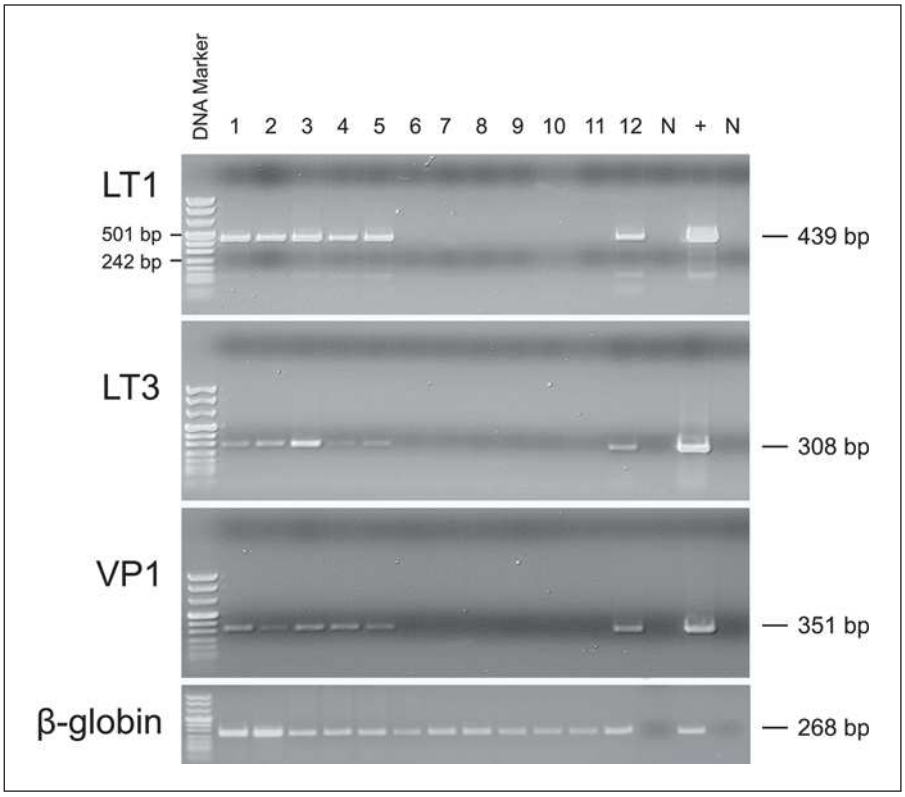

Fig. 1. Detection of MCPyV DNA in carcinoma in situ lesions and common warts of 2 EV patients (No. 3 and 5 in table 1). Genomic DNA from archival skin biopsies was amplified with primers specific for the LT1, LT3 or VP1 genes of MCPyV. DNA quality was checked by β-globin PCR to determine the presence of PCR-amplifiable DNA. The PCR products were separated on 1.5% agarose gels. DNA marker: low-mass DNA Molecular Weight Marker VIII (Roche; fragment lengths in base pairs are indicated). Lanes 1–5: 5 Bowen-type carcinoma in situ lesions of patient No. 3. Lanes 6 and 7: 2 Bowen-type carcinoma in situ lesions of 2 different immunocompetent patients. Lanes 8 and 9: 2 invasive SCC of 2 different immunocompetent patients. Lanes 10 and 11: 2 common warts of 2 different immunocompetent patients. Lane 12: common wart of EV patient No. 5. N = Negative (H_2O) control; + = MCPyV-positive, sequence-confirmed MCC control.

tients [16]. In contrast, out of 19 skin neoplasms from congenital EV patients, MCPyV was present in 16 lesions (84.2%; $p < 0.0001$, Fisher's exact test) (table 1).

In SCC and in actinic keratosis, we never detected MCPyV DNA. MCPyV DNA was also not detected in normal skin samples from healthy individuals (n = 7), but in 1 out of 17 psoriatic skin samples. This single MCPyV-positive psoriasis patient had also been previously diagnosed with MCC [20].

Detection of MCPyV in a Patient with AEV

EV-like symptoms have also been observed in the setting of immunosuppression, and this entity was suggested to be called AEV [12]. In addition to the congenital EV patients analyzed in this study, we identified 1 patient (No. 7 in table 1) who had developed EV-like symptoms during immunosuppressive treatment with azathioprine for Crohn's disease, consistent with the diagnosis of AEV. The histopathological findings for this patient were consistent with flat warts resembling EV (table 1).

We were able to analyze 2 common warts (verrucae vulgares) from patient No. 6. MCPyV DNA was detected in both lesions from this AEV patient. The MCPyV sequences showed 99% homology with the MCC350 virus genome (gb|EU375803.1 Merkel cell polyomavirus isolate MCC350) which had been identified in MCC. DNA of BKV, JCV, KIPyV or WUPyV was not detected in any of the samples (not shown). In both common warts, we found HPV-8 DNA, consistent with EV HPV infection under immunosuppression and, thus, with the diagnosis of AEV (table 1).

Discussion

In this study, we found that all congenital EV patients were harboring MCPyV DNA in their benign and malignant skin lesions, sometimes at different anatomic locations within the same individual. MCPyV was also detected in one patient with AEV [12]. Our findings indicate a general – inherited or acquired – susceptibility of EV patients to MCPyV infection. MCPyV was always present in conjunction with EV HPV types.

The high detection rate of MCPyV in benign and malignant skin lesions of EV patients is remarkable, as opposed to the low prevalence of MCPyV in NMSC of immunosuppressed and immunocompetent patients not suffering from EV. Despite the high seroprevalence of MCPyV in adults [24, 25], viral DNA is present only in a subset of

epithelial skin neoplasms. We and others found MCPyV DNA in up to 10% of carcinomas in situ of immunocompetent and immunosuppressed patients [15–18]. Intriguingly, approximately 27% of the European population show a polymorphism in one of the *EVER* genes [26]. Impaired *EVER* function in carriers of such polymorphisms could be an explanation for an increased susceptibility to MCPyV infection in a subset of the population. MCPyV DNA was not detected in actinic keratosis, in invasive SCC and in normal skin [16]. In contrast to MCPyV, EV HPV is frequently present in NMSC lesions [27].

In line with our findings, MCPyV DNA has recently been detected in one EV patient suffering from MCC [28]. As the risk to develop MCC is increased in immunosuppressed patients and EV seems to compromise the defense mechanisms against viral infections, EV might facilitate the development of MCC. However, it still remains to be determined if EV patients are more prone to the development of MCC. Alternatively, MCPyV as well as EV HPV might persist in a 'dormant' state in isolated cells and become reactivated under conditions of immunosuppression, as described for other members of the polyomavirus family.

EV results from a genetically determined defect in cell-mediated, cutaneous immunity [9]. Inactivating mutations in two adjacent genes on chromosome 17q25, *EVER1 (TMC6)* or *EVER2 (TMC8),* have recently been identified in EV patients [2]. It has been hypothesized that these genes play a role in regulating the distribution of zinc in the cell nucleus. Zinc has been shown to be a necessary cofactor for many viral proteins, and the activity of *EVER1/EVER2* appears to restrict the access of viral proteins to cellular zinc stores, limiting their growth [29]. Therefore, EV can be considered a result of a defect in cutaneous immunity that leaves afflicted individuals susceptible to persistent infection not only with EV HPV [9], but also with MCPyV. EV may not be the only skin disorder with an increased susceptibility to EV HPV and MCPyV. Both EV HPV type 5 and MCPyV have recently been detected in psoriatic skin lesions [20, 30–32]. In contrast to EV, there is only a weak association of MCPyV with psoriasis. Interestingly, the susceptibility locus for psoriasis (PSORS2) has been mapped to the short arm of chromosome 17q, which also contains the EV1 susceptibility locus [2, 33]. In a similar manner, another potential susceptibility locus for psoriasis was found on chromosome 2, near the EV2 susceptibility locus [31, 34]. Whether these genetic loci are also susceptibility loci for MCPyV infection, remains to be studied.

When we tested the skin biopsies of all EV patients by immunohistochemistry, using a monoclonal antibody directed against the large T protein of MCPyV [23], we detected a signal in isolated keratinocytes of some patients. As suggested before, MCPyV copy numbers are probably low in these skin lesions, hampering immunohistochemical detection of the virus [16, 23, 30]. Although speculative, MCPyV might infect single cells which then undergo neoplastic transformation and give rise to a malignant tumor. The tumor might become independent of the virus, which could persist in a 'dormant' state in isolated cells, similar to other members of the polyomavirus family such as BKV and JCV [35]. Sequence analysis did not reveal mutations of the amplified MCPyV large T gene in EV-associated skin neoplasms. This is in contrast to MCC, where truncating mutations of the MCPyV large T gene were identified [36]. Still, the absence of mutations of the MCPyV large T gene in EV-associated skin lesions does not exclude a pathogenetic role for MCPyV. There may be alternative pathways by which MCPyV may contribute to the pathogenesis of NMSC in EV patients.

Apart from pathogenetic aspects, the presence of MCPyV and EV HPV in all our EV patients could have diagnostic impact since EV is the only disease that is known so far to consistently harbor both viruses. The detection of EV HPV and MCPyV simultaneously present in one or several lesions suspicious for EV may serve as an adjunctive diagnostic marker for this rare genodermatosis. This association is of particular importance since *EVER* mutations have only recently been identified and the spectrum of *EVER* mutations in EV has still to be defined implicating a need for additional diagnostic criteria.

The presence of MCPyV in all EV patients and the oncogenic properties of MCPyV may suggest a pathogenetic model of how MCPyV could contribute to the cancerous progression of benign to malignant skin lesions, potentially in synergy with HPV. However, the strong association of EV HPV and MCPyV with EV does not prove a causative role per se, but rather suggests that these viruses contribute to the development of NMSC. To establish a causative role, several criteria have to be fulfilled [37]. Further studies are needed to confirm consistency of the association, i.e. reproducibility of the findings by various investigators and, ideally, also different methods.

In summary, EV may be redefined as a genodermatosis with a unique susceptibility to infection with both EV HPV and MCPyV, which may act as synergistic carcinogenic cofactors in the development of NMSC in EV. In spite of the small cohort size, the present study suggests that EV could be the first genetic predisposition towards MCPyV infection and represent a model not only for HPV-associated, but also for MCPyV-associated cutaneous oncogenesis.

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